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The Biocompatibility and Bioactivity of Biodentine in Contact with Cementoblast Cells

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The Biocompatibility and Bioactivity of Biodentine® in Contact with Cementoblast Cells

ABSTRACT

Introduction: Biodentine® is a dental material used for perforation repairs, root-end fillings, and direct pulp-capping. Published reports indicate Biodentine® is biocompatible but its effect on cementoblasts or relationship to ion release has not been determined; furthermore, different cytotoxicity tests have not been compared for applicability. This study evaluated three strategies for testing Biodentine® cytotoxicity towards immortalized cementoblasts (OCCM cells). **Methods:** Biodentine® disks were fabricated in 96-well plates. Disks were eluted with media: short term; 48 hrs; long term; and daily for 19 days; pH was measured in a $C0_2$ atmosphere, and calcium ion concentration determined. OCCM cells were plated onto the Biodentine® disks and empty (tissue culture plastic control) wells and grown for 48 hours. Flow cytometry, Picogreen DNA assay, and direct staining with Crystal Violet or MTI, counting by microscopy and cell morphology were evaluated. **Results:** Significant media pH and calcium ion changes in media exposed to Biodentine® with few or no elutions were evident, but approached control values within hours. Staining with MTI and direct counting was the most reliable method for cell quantification on the Biodentine® surface. Crystal Violet and MTI staining showed significantly fewer cells with an altered morphology on the Biodentine® surface, continuing even after 19 elutions. **Conclusion:** Freshly set Biodentine® demonstrates long-term cytotoxicity towards immortalized

cementoblasts; this was only initially associated with media changes in pH and calcium ion levels, suggesting surface topology could have a negative effect on cells.

INTRODUCTION

An ideal endodontic restorative material is biocompatible. Thus, it should be non-toxic, non-cytotoxic to exposed cells, non-carcinogenic, insoluble in tissue fluids and dimensionally stable (1, 7). Therefore, preferred endodontic materials are biologically neutral or better, can promote cellular repair (2). Materials used as root-end filling materials or used to repair root perforations may extend their biological effects to the periradicular tissues, including cementoblasts. Cementoblasts are of particular interest because their viability is critical to healing and cementogenesis of the root surface.

Mineral trioxide aggregate (MTA}, a radiopaque mixture of tricalcium silicate, tricalcium aluminate, calcium silicate, and tetracalcium aluminoferrite, has become the preferred material for perforation repair and root-end fillings due to its ability to be hard tissue conductive, and its biocompatibility towards surrounding tissues (3,4,5). Additionally, it possesses good antimicrobial activity (6), in part related to the significant release of calcium and hydroxyl ions during setting, which elevates the local pH (18). Despite its many advantages, MTA exhibits several unwelcomed physical and chemical properties, particularly poor handing properties, a prolonged setting time and a potential for staining tooth structure (7, 8, 14, 15, 16).

Biodentine® (Septodont, St-Maur-des-Fosses, France) is a commercial alternative to MTA consisting of a powder containing tricalcium silicate, dicalcium silicate, and calcium carbonate, with zirconium oxide as a radiopacifier. It is mixed with a water-based liquid containing calcium chloride as a setting accelerator and a hydrosoluble polymer that serves as a water-reducing agent. Biodentine® is manufactured for use as a perforation repair material, root-end filling material, and as a direct pulp-capping agent (9, 10). Reported benefits of Biodentine® include its ease of handling, high viscosity, mineralized bridge formation and short setting time (12 minutes) (10). Research has shown Biodentine® to be as effective as MTA in stimulation of hard tissue formation, indicating its justification for use in root repair and root-end filling (25, 26).

In a rat model, Biodentine® introduced to subcutaneous tissues showed an initial inflammatory response that was followed by biocompatible acceptance of the material after two weeks of tissue contact (11). Another study assessing the viability of embryonic fibroblast cells in direct contact with Biodentine® or MTA reported a similar cytotoxicity for the two materials. (12). While these studies support Biodentine® being a biocompatible material, to date, there have been no reports on the cytotoxic effects of Biodentine® on periradicular cells, in particular cementoblasts.

In principle, direct determination of cell numbers by counting is the best strategy for the determination of a material's biocompatibility when exposed to a certain cell type (20). However, manual counting of cells, e.g., using a hemacytometer, is time consuming. Flow cytometry may be an ideal alternative because it allows for an automated, rapid, inexpensive and sensitive direct quantitative analysis of cell number

and viability (9). Colorimetric metabolic assays are popular for assessing cell viability and cytotoxicity due to their ease of use and adaptability for high sample studies. The rationale is that the amount of a colored metabolism-based enzymatic reaction product will be proportional to cell number. However, a confounding effect can result if the tested materials influence cellular metabolism. An alternative strategy for indirect cell quantification is to measure DNA based on the assumption of a constant average DNA content, and the PicoGreen DNA quantification assay has been used efficiently to quantify DNA in small tissue samples (17). Although routine cytotoxicity studies focus typically on the effects of released materials in solution, prior research has shown that the surface of a material on which a cell can attach, migrate and differentiate can have a profound effect on cell fate (21, 22). To measure such effects, cytotoxicity assays must be shown to be compatible and usable with surface growth of cells.

The purpose of this study was to evaluate different cytotoxicity assay methods for determination of cell numbers on material surfaces, and to determine the cytotoxic activity of Biodentine® towards an immortalized cementoblast cell line (OCCM) after different periods of surface elution in conjunction with determination of hydroxyl ion (via pH) and calcium ion release.

Materials and Methods

pH and Calcium ion release from Biodentine into tissue culture media

The effect of direct continuous contact with set Biodentine® on the pH and calcium ion content of tissue culture media in a 5% C02 atmosphere was determined

over a 48 hour period. (The fabrication of the disks is described in the supplement). Media was placed in an equal number of empty wells in the same plates to serve as controls.

With the plate in the incubator to maintain buffering C02, a calibrated microelectrode was used to measure the media pH at 30 min, 1 hr, 2 hr, 4 hr, 6 hr, 8 hr, 10 hr, 14hr, 24 hr, 36 hr, and 48 hr. After measurement, 200 µL of media was removed; for disk media, aliquots were placed in a microfuge tube and centrifuged for one minute. Supernatant (100 µL) was removed, mixed with 400 µL of saline (0.9% sodium chloride), and the Ca^{2+} concentration measured with a calibrated electrode (Orion Calcium Ion Selective Electrode, Thermo Fisher Scientific, Waltham. MA).

Hydroxyl ion (as pH) and Calcium ion release from Biodentine® into tissue media with replacement and effect on OCCM growth over 20 days

Biodentine® disks were fabricated 20, 17, 13, 10, 7, 6, 5,4, 3, 2, and 1 day prior the last media change. On each of the designated days, two sets of 5 Biodentine® disks were fabricated in a 96-well plate, with the same number of plastic wells being used as controls. Removed media was transferred to a new 96 well plate (to avoid disk contamination), the pH was measured and then the sample was diluted (4:1 with sterile 0.9% saline) and frozen for later calcium ion release measurement.

The OCCM cell line used was previously described in detail by D'Errico et al (13), and kindly provided by Dr. Anne Tran in the Laboratory of Oral Connective Tissue

Biology, NIH/NIAMS. OCCM cells were maintained in supplemented DMEM in a humidified atmosphere of 5% $C0_2$ in air at 37°C.

Day 1 disks were allowed to cure overnight, at which time media was removed from the disks for the final day's measurements. One confluent T75 flask of OCCM cells was used to prepare an 80 ml cell suspension at a density of 62,500 cells/ml, as determined by cell counts using a hemacytometer. Initial viability was >95%, as determined by trypan blue staining. Biodentine® disks and control plastic wells were plated with 200µ1 (12,500 cells) of OCCM cell suspension. This density was established previously in pilot experiments to provide logarithmic growth over a 48 hour period.

After 48 hours of growth, the media was removed and wells from each plate were prepared for MTT metabolic staining (5-6 from Biodentine and 5-6 from plastic) and for crystal violet staining per published protocols (23, 24). The individual wells were photographed using a Zeiss (Oberkochen, Germany) Stemi 508 (5x) microscope and a Zeiss microscope equipped with an AxioCam MRM camera (1Ox and 40x). Cells were quantified using Zeiss software (AxioVision SE64 4.9.1).

GraphPad Prism 6.0 software (GraphPad Software, LaJolla, CA) was used for the statistical analysis. Changes in pH and Ca ion levels were compared by one and two way ANOVA. Alpha was 0.05.

RESULTS

Exposure of media to Biodentine® over a 48 hr period showed a significant initial increase in pH as compared to plastic (Figure 1a). The pH rose rapidly

to a peak of pH 9.81±.0.41 (sem) by 14 hours, and then declined to control levels (7.80±_0.18) by 24 hours; thereafter remaining unchanged. Calcium ion concentration showed a parallel dramatic rise, also peaking hour 14 (3.36±. 0.05x10^3 ppm) and then declining, but not to the level of the control (54.6±_4.7 ppm), remaining at or above 0.78±. 0.22x10^3 ppm (Figure 1b).

The pH of media exposed to Biodentine® over a 20 day period with daily media changes, closely followed that of the control throughout the experiment, and showed no significant change (Figure 2a). The initial high calcium ion release declined after Day 1 and was not significantly different from control levels (54.6±_4.7 ppm) at Day 2 (Figure 2b).

Evaluation of flow cytometry and Picogreen assays for quantification of cells revealed incompatibility with the test material (see Supplemental information). Quantification of OCCM cells per unit area by microscopy after staining with MTT was selected as the most reliable assay method for quantification of cell numbers (see Supplemental information). Cells grown on plastic increased from 0.39 x10^3 cells/mm^2 to 0.62±_0.05x10^3 cells/mm^2 during 48hrs of growth. In comparison, there was an initial marked 87% decrease in the number of cells present on Biodentine® disks with no media elution after 48hrs (0.05_±0.01x10^3 cells/mm^2). A significant decrease in the number of OCCM cells grown on Biodentine® disks was also demonstrated with crystal violet staining, but quantification was less reproducible due to background staining In addition to fewer cells. Both the crystal violet and MTT staining showed a marked change in morphology of the cells grown on Biodentine® disks versus those grown on plastic (Figures 3a-d). Cells on plastic showed a more spread out, fibroblast-like

appearance with granular, mainly perinuclear staining, whereas cells grown on Biodentine® initially had a mix of more rounded and highly elongated growth and more intense staining (Figures 4a-c). By Day 20, the morphology was beginning to resemble that on plastic, but still with a high proportion of elongated cells.

DISCUSSION

Consistent with other reports (20), this study revealed challenges in measuring cell proliferation on bioactive surfaces. Pilot studies revealed that Biodentine® quenched PicoGreen fluorescence, precluding use of PicoGreen as an assay. Pilot studies using flow cytometry to count cells directly demonstrated unreliable cell harvesting with trypsin and a very high particulate release from the freshly set Biodentine® (see supplement). Additional pilot studies demonstrated that Biodentine® appeared to elevate cellular MTT staining, potentially resulting in misleadingly high estimates for viability after solubilizing and quantifying dye spectrophotometrically. Direct counting of formalin-fixed stained cells was found to be the most reliable method, and also provided information on cell morphology. In comparison to crystal violet staining, microscopic evaluation of formalin fixed cells stained using the MTT metabolic assay was found to give less background, and was also restricted to viable (metabolically active) cells. However, Crystal Violet staining appeared to give the best preservation of morphology.

Prior studies have reported the release of calcium and hydroxyl ions into solution and associated pH changes in fluids exposed to Biodentine® (18, 19); however, these studies used limited time points and did not assess any correlation with potential

cytotoxicity. In contrast, our study evaluated ion release and pH changes (both short and long-term) in an effort to correlate this data with changes in cementoblast morphology and quantity.

Despite daily media changes, Biodentine® appeared to be highly cytotoxic to cells over a three week period, limiting growth and resulting in a different morphology when compared with cell growth on plastic. This cytotoxic effect was not limited to OCCM cells, with prior pilot studies displaying similar effects being observed over a 48 hr growth period with MG63 osteosarcoma cells and TIME immortalized endothelial cells (see supplemental information).

Under clinical conditions, it is assumed that due to clearance by extracellular fluid flow, the periapical tissues would come into contact with progressively lower concentrations of the leachable cytotoxic compounds (primarily calcium and hydroxyl ions) produced by the setting reaction of the Biodentine®. To simulate clinical interaction with the material, cytotoxicity was analyzed after different periods of elution ranging from 1 to 20 days using changes of fresh media tissue culture. Significant OCCM cytoxicity was evident with direct contact with Biodentine® samples with few or no elutions. Under the assay conditions, such low-elution samples would produce the maximum bolus of released ions that cells would encounter at the material surface. As the number of elutions increased, the cytotoxicity of Biodentine® decreased but the decline did not correlate with changes in the pH or calcium ion concentration in the media. The hydroxyl ion levels were essentially unchanged from control, and calcium ion levels had returned to normal after just one day of elution, but cell growth on

Biodentine was only 15% that on plastic at this time. Even after 20 daily elutions, growth of OCCM cells on Biodentine was still 35% lower than on plastic.

The results of this study suggest that even after extensive elution, the Biodentine® surface either inhibited cell proliferation, and/or cells failed to attach efficiently and were readily lost during the initial wash steps of the cell harvesting procedure. However, it is important to consider that although inhibition of cell growth is scored as cytotoxicity in traditional testing, it is conceivable that Biodentine® could be inducing the immortalized OCCM cells into a more differentiated, and non-proliferative state, which would be beneficial. Examination of the pattern of gene expression in OCCM cells grown on Biodentine® would be required to test this possibility.

CONCLUSION

As measured by MTT and crystalviolet staining and cell counting assays, Biodentine® exhibited cytotoxicity towards immortalized cementoblasts. This was likely the result of cell death, inhibition of cell growth (whether due to cytotoxicity or induction of differentiation) and/or failure of cells to attach. The increase in pH and calcium ion release from Biodentine® could contribute to the initial cytotoxicity, but later inhibitory effects could be due to surface topography. Since Biodentine® could have different effects on cells in the periradicular region; it therefore has the potential to influence local tissue type formation.

No competing financial interests exist. The views expressed in this manuscript are those of the authors and do not necessarily reflect the official policy of the Department of Defense, Department of Army, US Army Medical Department or the US Government.

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Figure Legends

Figure 1. Effect of 24hr cured Biodentine® on media pH and calcium ion concentration over a 48hr period.

(A) Media pH. The pH of media in a C02 atmosphere exposed to 24hr cured Biodentine® or tissue culture plastic was determined over a 48 hr period. Error bars show sem (n=3 independent experiments ; 9 replicates per time point). Two-way ANOVA showed a significant effect for time, treatment and interaction (<0.0001). At 14 hr (''') the pH of media exposed to Biodentine® was significantly greater than at 0.5, 1.25 or 2hrs (Sidak's multiple comparison's test; p<0.004) or at 24, 36 Or 48 hrs (p<0.005), and significantly greater that media on plastic (p=0.001). Media on plastic showed no significant differences with time (p>0.05; overall mean 8.02+0.03 (sem)). No other significant differences were seen.

(B) Media calcium ion concentration.The calcium ion concentration (ppm) of media in a C02 atmosphere exposed to 24hr cured Biodentine® or tissue culture

plastic was determined over a 48 hr period. Error bars show sem (n=3 independent experiments; 5-6 replicates per time point). Two-way ANOVA showed a significant effect for time, treatment and interaction ($p<0.0001$). In media exposed to Biodentine®, by 3.25hr the calcium ion level was significantly higher than media on plastic ($p=0.0008$), and continued to rise in value through 14 hrs (#), but with no significant difference between 5.5, 7.5, 10 and 14 hrs ($p>0.05$), consistent with a broad plateau. The concentration at 24, 36 and 48 hrs (*) was significantly lower than at 14hrs ($p<0.017$), but there was no significant difference in concentration between 24, 36 and 48hrs ($p>0.05$). Media on plastic showed no significant differences with time ($p>0.05$; overall mean 54.6+3.1 ppm (sem)). The concentration in media exposed to Biodentine® at 24 and 48 hrs was significantly higher than media on plastic ($p<0.009$).

Figure 2. Effect of daily elution of 24hr cured Biodentine® on media pH and calcium ion concentration. Values for Day 0 are the overall means for pH (8.02) and Calcium ion concentration (54.6 ppm) taken from Figure 1. Days of elution represent the number of days the media was changed over the Biodentine® (with measurements taken from the last change).

(A) Elution media pH. Two-way ANOVA showed a significant effect for treatment ($p=0.0004$) and days of elution ($p<0.0001$), but not interaction (p0.05). However, no individual differences by day were detected between Biodentine® and plastic-

treated media (Sidak's multiple comparisons test; p>0.5), consistent with a weak overall trend to higher pH from a modest low at Day 1.

(B) Elution media Calcium ion concentration (ppm). One-way ANOVA comparison of calcium ion concentrations showed a highly significant effect for days of elution (p<0.0001). Error bars show sem (treating each daily media change as independent sampling across days). The concentration Day 1 was significantly higher than all other days (Tukey's multiple comparisons test; p<0.0001), while the concentrations at all other days were not significantly different from each other, or from the mean media on plastic concentration (blue symbol Day 0) (p0.05).

(C) Cell growth on media eluted Biodentine® as a percentage of tissue culture plastic control. OCCM cells were plated at a density of 12,500 cells per well, grown for 48 hrs, and quantified by MTI staining, fixation, and counting by inverted reflected light microscopy and software analysis. Error bars are SEM. The straight line shows a linear regression analysis ($r2 = 0.66$) with corresponding elutions from a low of 8.6% (the number of cells compared to the plastic control wells) on Day 1, to 65% by Day 20.

Figure 3

Image A: Cementoblasts grown on plastic (5X initial magnification) stained with Crystal Violet.

Image B: Cementoblasts grown on Biodentine® (5X initial magnification) Day 1, stained with Crystal Violet. Note the decrease in quantity and altered morphology.

Image C: Cementoblasts grown on plastic (5X initial magnification) stained with MTI metabolic assay.

Image D: Cementoblasts grown on Biodentine® (5X initial magnification) Day 1, stained with MTI. Note the decrease in quantity and altered morphology.

Figure 4

Image A: Cementoblasts grown on plastic, stained with MTT metabolic assay (20X initial magnification).

Image B: Cementoblasts grown on Biodentine®, stained with MTT metabolic assay, Day 1 (20X initial magnification). Note the altered morphology of the cells compared to those grown on plastic (very rounded).

Image C: Cementoblasts grown on Biodentine®, stained with MTI metabolic assay, Day 20 (20X initial magnification). Note the different morphology compared to Day 1 cells grown on Biodentine®. The Day 20 cells (20 media changes) more closely resemble those grown on plastic, but dark stained, elongated cells were still present.

Supplemental Information

Additional Materials and Methods:

Biodentine disk fabrication: Biodentine® (Lot # B09846) disks were fabricated in a laminar flow hood using sterile technique. Biodentine® powder and liquid were mixed in strict compliance with the manufacturer's instruction. Approximately equal amounts of Biodentine® were placed in wells in a 96-well plate, and compacted with a sterile flat-bottomed steel rod to a height of approximately 1mm. Following plating, the 96-well plates were placed overnight in a tissue culture incubator (37°C, 5% C02, 100% relative humidity) to allow the material to set. Disks were irradiated with UV for 30 minutes prior to use.

Tissue Culture: The media used was Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal calf serum (FCS), L-glutamine (2mM), and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). Prior to removal, the media was treated with 10 m of phosphate-buffered saline (PBS) to eliminate calcium and protein. After removal of the PBS, 2.5 ml of Trypsin was added to initiate the detachment of the cells and the media was placed in the incubator for 2 minutes.

Flow cytometry: OCCM cells were plated on cured Biodentine disks that had been eluted daily for 0-14 days, and grown for 48 hrs. In parallel, OCCM cells were plated in plastic wells as a control. Cells were harvested by treatment with trypsin for increasing periods of time, and remaining cells identified by Crystal Violet staining. Media drawn off Biodentine and harvested cells were analyzed

using flow cytometry (Accuri c6 Flow Cytometer) and CFlow software. The distribution of cells was analyzed by Sytox Green staining. The volume of liquid counted per cycle ranged between 400-1000 µL and was determined from prior pilot studies, based upon the need to achieve an adequate cell count number. The resulting histograms were evaluated. Another pilot study compared media placed on plastic with media on non-eluted Biodentine® disks with the resulting histograms evaluated.

PicoGreen® protocol: On the day of use, an aqueous working solution of the PicoGreen® reagent (Quant-iT PicoGreen dsDNA Assay Kit; Molecular Probes, Darmstadt, Germany) was prepared by making a 200-fold dilution of the concentrated DMSO solution in TE buffer (10 mM Tris-HCL, 1mM EDTA, pH 7.5). 1.0ml of the aqueous working solution of PicoGreen® was pipetted into each well and incubated for 2 to 5 minutes at room temperature, protected from light. After incubation, the sample's fluorescence was measured using a spectrofluorometer microplate reader.

MTT protocol:

Twenty four hours after fabrication the Biodentine® disks (per experiment: 11 plates, 5-6 disks) were covered with 250 µL supplemented DMEM, and returned to the incubator. An equal number of plastic wells were also covered with the same media to serve as controls at each time point (Suppl. Figure 2). On day 0, the wells were plated with 200µ1 of OCCM cell suspension (i.e., a density of

12,500 cells per well). This density was previously established in pilot experiments to provide logarithmic growth over a 48 hour period.

After 48 hours of growth, the media was removed by aspiration, and wells washed with 200 µL of PBS (phosphate-buffered saline). MTI solution (100 µL; 0.1% MTI, 0.49mM $MgCl_2$, 2.5mM $CoCl_2$, 125mM sodium succinate, 50mM Tris-HCl pH 7.4) was added to each well and the plate was incubated for two hours (determined to be optimal in pilot experiments). Then 100 µL of formalin (0.2 M Tris, 4% formalin, pH 7.7) was added to each well and the cells were fixed for 5 minutes. The liquid were aspirated off and wells were rinsed with 200 µL of water, aspirated dry and photographed.

CV protocol:

The media was removed from wells by aspiration and ice-cold PBS was placed in each of the wells for 5 min. The PBS was changed once and the wells were aspirated dry and 100% ice-cold ethanol was placed on the cells for 10 minutes. The ethanol was removed and Crystal Violet was placed for 10 minutes (still on Ice). The Crystal Violet was removed and the wells were washed multiple times with cold water, allowed to dry and photographed.

Supplemental Results:

Figure 1a shows a flow cytometry histogram generated from fresh media (no cells), no gating, with 200 µL being analyzed. Only small particles are present,

clustered near the origin. Figure 1b shows media taken from a Biodentine well, no gating, 600 µL volume with 200 µL being analyzed. The analysis stopped after -90 µL due to the maximum number of measurements (1,000,000) being reached. The distribution overlapped with the location of cells (particularly dead cells), and too few cells were present in the small volume to count reliably. The number of particulates formed by media contact with Biodentine® only declined to acceptable levels after several days of elution.

A further issue was the lack of quantitative release of cells from the surface by trypsinization (not shown), likely due in part to the high levels of calcium present interfering with the effectiveness of the EDTA

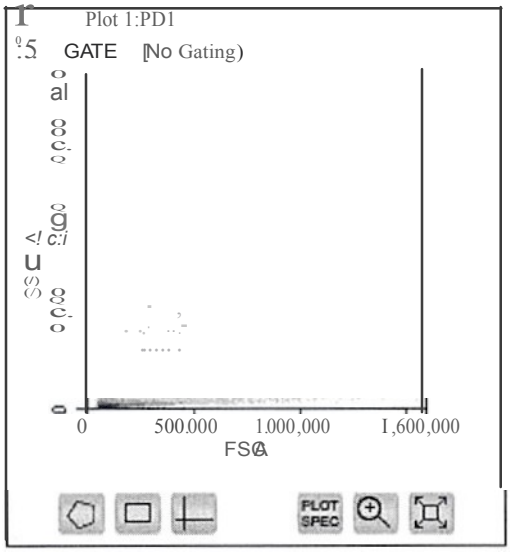

Figure 1a: Media on plastic.

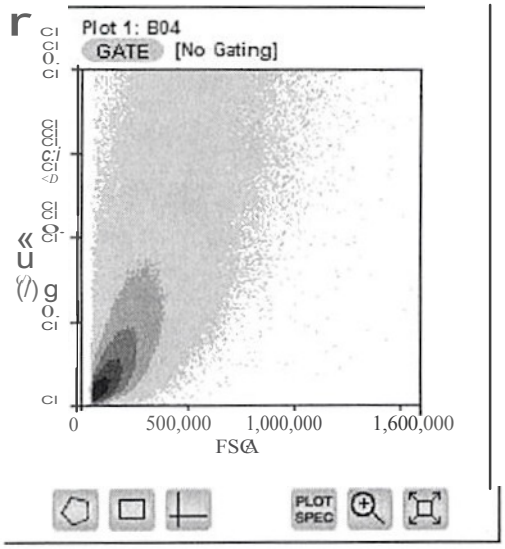

Figure 1b: Media on non-eluted Biodentine® disks.

Therefore, flow cytometry to measure cytotoxicity of Biodentine® was considered unreliable.

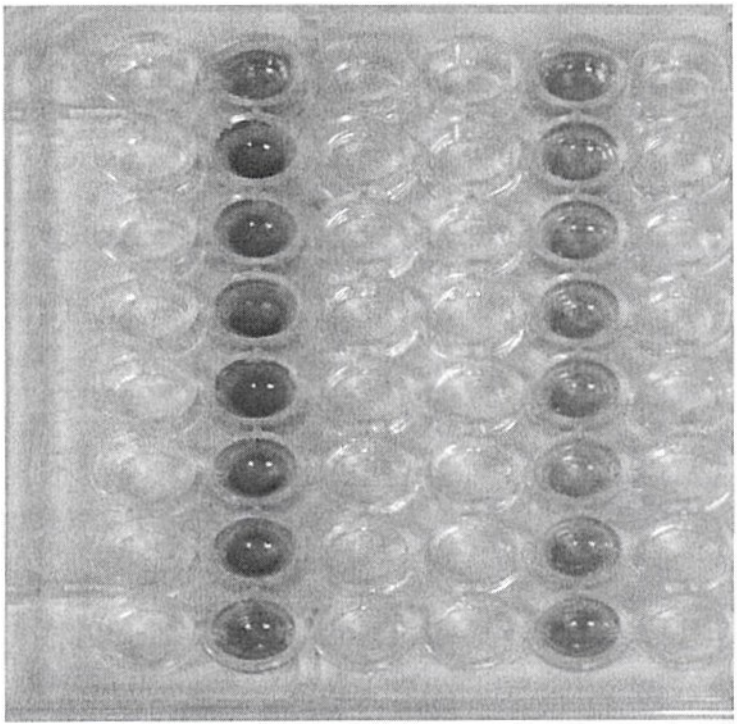

Figure 2

Other pilot studies:

Pilot experiments demonstrated that Biodentine® initially elevates markedly the cellular MTT staining in MG-63 and TIME immortalized endothelial cells, resulting in misleadingly high estimates for viability when the dye is solubilized and quantified spectrophotometrically (Gaudry, Horspool and Dickinson; unpublished observations) . See Figures 3a-d.

24 hour results for MG-63 cells and endothelial cells:

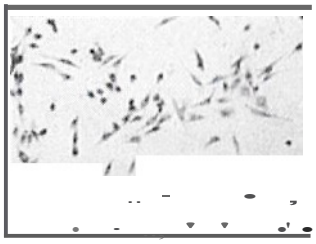

Figure 3a: MG-63 cells on plastic

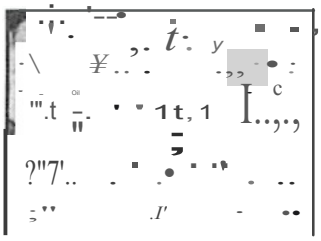

Figure 3b: MG-63 cells on Biodentine®

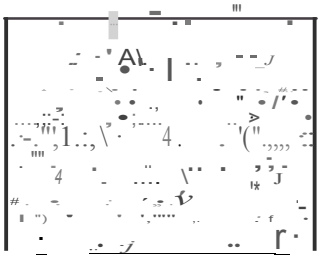

Figure 3c: Endothelial cells on plastic

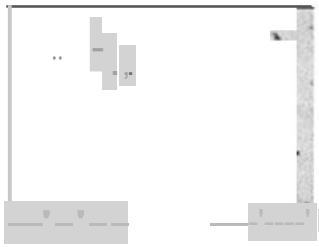

Figure 3d: Endothelial cells on Biodentine®